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The goals of our research are to investigate the molecular mechanisms controlling luminescence gene expression of the symbiotic, light-organ bacterium Vibrio fischeri; and to identify and investigate the regulation of other symbiosis functions in this marine bacterium. During the past year, we have: 1) completed studies in Escherichia coli cya and crp mutants on expression of the luxR gene that demonstrate cAMP and CRP activate transcription of luxR and that the LuxR protein, possibly working in concert with autoinducer, represses transcription from the luxR promoter (transcriptional negative autoregulation); 2) isolated mutants of V. fischeri apparently deficient in adenylate cyclase and CRP, and demonstrated the requirement for cAMP and CRP in autoinduction of luminescence and in iron regulation of luminescence; 3) initiated studies in E. coli of the role of autoinducer in luxR negative autoregulation; and, 4) initiated work toward development of a gene transfer system for V. fischeri.

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PRINCIPAL INVESTIGATOR: Paul V. Dunlap

CONTRACTOR: New Mexico State University
Las Cruces, NM

CONTRACT TITLE: *Vibrio fischeri* Symbiosis Gene Regulation

PROJECT PERIOD: August 15, 1987 -- August 14, 1988

RESEARCH OBJECTIVES:

The goals of our research are to investigate the molecular mechanisms controlling luminescence gene expression of the symbiotic, light-organ bacterium *Vibrio fischeri*; and to identify and investigate the regulation of other symbiosis functions in this marine bacterium.

PROGRESS (Year 1):

1. Regulation of *V. fischeri lux* gene expression in *E. coli*.

A. Transcriptional control of *luxR* expression by cAMP-CRP and LuxR

Evidence to date indicates that the *luxR* gene product is the transcriptional activator of the *V. fischeri luxICDABE* genes (genes for autoinducer synthesis and luminescence enzymes). As such, a knowledge of the regulatory factors that control *luxR* expression is essential for understanding *lux* gene regulation both in general terms and in detail. Previous studies had indicated that cyclic AMP (cAMP) and cAMP receptor protein (CRP) are required for induction of the *luxICDABE* genes in *E. coli*, as monitored with luciferase (*luxAB* gene product), and that cAMP-CRP appeared to function in the *lux* regulon by activating transcription from the *luxR* promoter, as monitored with β-galactosidase from a Mu dI (*lacZ*) insertion in *luxR*. However, these studies were conducted in the absence of a functional *luxR* gene (due to insertional inactivation by Mu dI) and with a single *lacZ::luxR* fusion.

To address these caveats and to better understand the mechanism of transcriptional control of *luxR*, a *luxR* complementation plasmid (pPD749) was developed by sub-cloning *luxR* under control of the tac promoter to provide LuxR in trans under control of IPTG (Fig. 1A). Furthermore, additional *lacZ::luxR* fusions were generated using Mu dI1734, and four that mapped at widely separated positions in *luxR* (Fig. 1B) were examined in the complementation system.

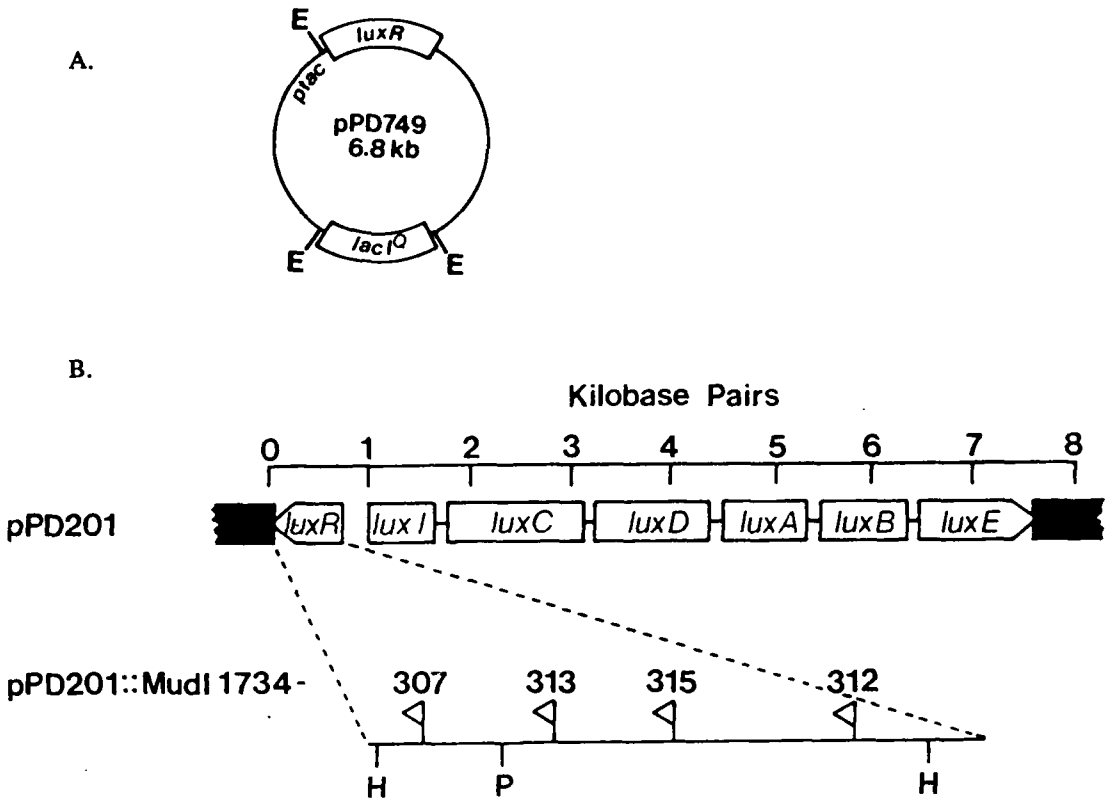

FIG. 1. Two plasmid *lux* complementation system. A) pPD749, containing *luxR* under control of the *tac* promoter and with the *lacI* gene for IPTG control of the *tac* promoter. B) Organization of the *lux* genes and map locations of the four *luxR*::Mu dI1734 (*lacZ*) fusions used to study *luxR* promoter activity. E, EcoRI; H, HindIII; P, PstI. The flags indicate the positions of the Mud I1734 insertions and point in the direction of *lacZ* transcription.

Studies with these recombinant *lux* plasmids in *E. coli* adenylate cyclase and CRP mutants (*cya* and *crp* mutants) revealed: 1) that in the presence of high levels of the *luxR* gene product (that is, LuxR expressed from pPD749), cAMP and CRP are no longer necessary for induction of luminescence and luciferase synthesis (i. e., transcription of *luxICDABE*); 2) that cAMP and CRP activate transcription from the *luxR* promoter regardless of the presence or absence of a functional *luxR* gene and regardless of the position of the *lacZ* fusion in *luxR*; and strikingly, 3) that the *luxR* gene product negatively autoregulates transcription from the *luxR* promoter. These results are interpreted as indicating that cAMP-CRP function indirectly in the *lux* regulon by activating transcription from the *luxR* promoter. This activation leads to an increase in the level of LuxR in the cells, thereby potentiating the system for autoinduction. These studies have led to a working model for *lux* gene regulation that includes this role for cAMP-CRP and the role of LuxR in *luxR* negative autoregulation (Fig. 2).

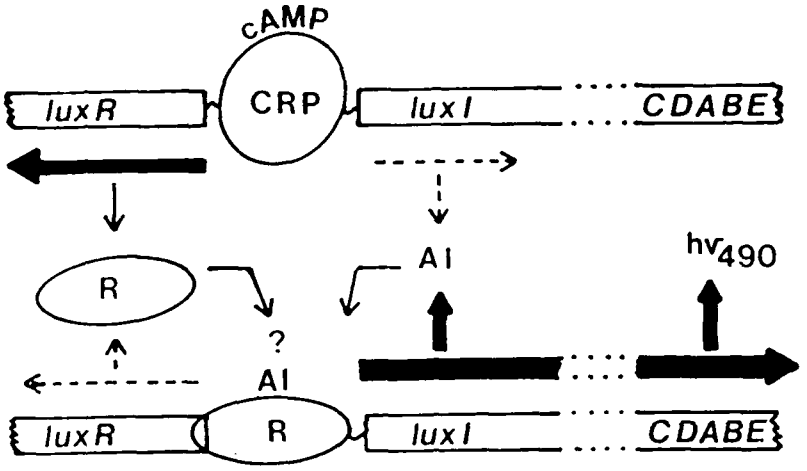

FIG. 2. Model for transcriptional regulation of the *V. fischeri lux* regulon. CRP and cAMP bind to the region between *luxR* and *luxI*, activate transcription of *luxR* (indicated by the heavy arrow) and decrease transcription of *luxICDABE* (indicated by the very light arrow). The activation of *luxR* transcription increases the concentration of the LuxR protein (R) to a sufficient level for interaction with the low concentration of autoinducer (AI) that has accumulated due to basal expression of the *luxI* gene and the resulting low activity of the *luxI* gene product. The LuxR protein and autoinducer then bind to a region upstream of the *luxICDABE* promoter, activating transcription of *luxICDABE*. This leads to increased levels of autoinducer and to induced levels of luminescence ($h\nu_{490}$).

(Note: The work described above was initiated in the laboratory of Dr. E. P. Greenberg, Cornell University, while I was a post-doctoral research associate. The work was completed at NMSU with ONR support.)

B. Role of autoinducer in *luxR* negative autoregulation

According to the current working model for *lux* gene regulation (Fig. 2), autoinducer plays an essential role in transcriptional activation of *luxICDABE*. It is not clear from the studies described above, however, whether autoinducer is actually involved in the observed transcriptional negative autoregulation of *luxR* expression, although the data suggest this. Cells in the above studies contained an intact *luxI* (autoinducer) gene, enabling them to produce autoinducer. Consequently, the presence and amount of autoinducer could not be controlled during the course of the experiments. To address the question of the role of autoinducer in *luxR* negative autoregulation, we are attempting to generate mutations in the *luxI* gene so that experiments similar to those described above can be conducted in the absence of autoinducer and with controlled amounts of autoinducer. To accomplish this, we are creating point mutations in the *lux* genes, using an in vitro hydroxylamine mutagenesis procedure with the cloned *lux* system, and then screening them using a two-plasmid complementation protocol to identify those mutations occurring in *luxI*. Progress to date includes development of a screening protocol and the acquisition of several possible *luxI* mutations, as determined by partially completed screenings.

2. Regulation of *lux* gene expression in *V. fischeri*

A. Regulatory mutants of *V. fischeri*

As described above, much of the existing information on cAMP-CRP control of luminescence has been derived from studies with *E. coli* containing recombinant *lux* plasmids. While the results of these studies provide a consistent and interpretable view of *lux* gene regulation, the fact that these studies were carried out in a heterologous background with multi-copy plasmids could pose problems due to possible artefacts in the observed regulation. Thus, in the absence of comparable studies with mutants of *V. fischeri*, the validity of these studies of cAMP-CRP control of *lux* gene expression in *E. coli* remains subject to question. At the time the work with *E. coli* was initiated, there were no cAMP and CRP mutants of *V. fischeri* with which to addess this problem.

Consequently, to resolve this problem, we have used a phosphomycin selection procedure to isolate spontaneous mutants of *V. fischeri* apparently deficient in adenylate cyclase (Cya) and CRP (Crp). The isolation procedure required several modifications to accomodate the growth requirements of *V. fischeri* and its high sensitivity to components of indicator media. Despite these problems, a successful procedure was developed. The isolated mutants exhibit several characteristics comparable to *cya* and *crp* mutants of *E. coli* and *Salmonella typhimurium*, including a pleiotropic carbohydrate negative phenotype and a decreased sensitivity to antibiotics thought to be transported by cAMP-CRP-controlled systems. Notably, compared to the wild-type strain, the mutants produce a very low level of luminescence and luciferase in the absence of added cAMP. Addition of cAMP to the growth medium, with or without added autoinducer, restores luminescence and luciferase synthesis in the Cya mutant to levels approaching those of the wild-type strain, but has no effect on luminescence or luciferase synthesis in the Crp mutant (see Fig. 3). Addition of autoinducer alone effects a small increase in luminescence and luciferase synthesis in both mutants. These results demonstrate that cAMP and CRP are required for the autoinduction of luminescence in *V. fischeri*, and they are very similar to results obtained with *E. coli cya* and *crp* mutants containing the *V. fischeri lux* genes on recombinant plasmids. Thus, these results confirm earlier conclusions for cAMP-CRP control of luminescence.

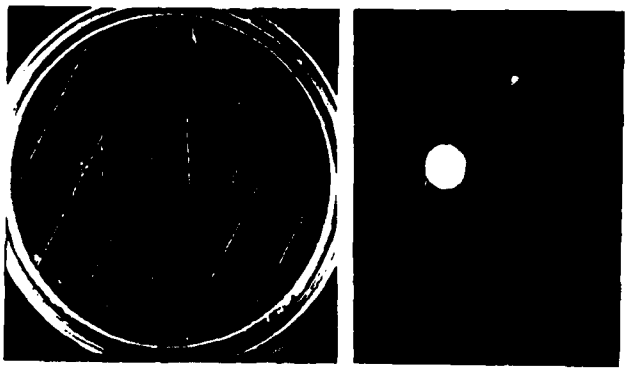

FIG 3. Luminescence response of *V. fischeri* Cya and Crp mutants to cAMP. (Left panel) Cya mutant on the left and Crp mutant on the right, photographed in the light approximately 4 hrs after addition of cAMP to cells. (Right panel) Same plate photographed in the dark.

Except for natural isolates of *V. fischeri* that are deficient in autoinducer synthesis, these Cya and Crp mutants are the first regulatory mutants of this species to be described. Their isolation should facilitate studies of *lux* gene regulation and other cAMP-CRP-controlled functions in *V. fischeri*.

For example, the use of these mutants has unexpectedly revealed a requirement for cAMP and CRP in iron regulation of luminescence. In *V. fischeri*, it has been shown by other investigators that excess iron can repress luminescence, and limitation of iron availability stimulates luminescence and luciferase synthesis while restricting growth. The mechanism for iron regulation of luminescence is not know. To determine if cAMP and CRP are involved in this iron regulation, we grew the Cya and Crp mutants of *V. fischeri* under iron-limited conditions by adding the iron chelator, EDDHA, to the growth medium. Addition of EDDHA was found to restrict the growth of both mutants (as it does in the wild-type strain), but it stimulated luminescence only in the Cya mutant and only in the presence of added cAMP. We interpret these results as indicating that the stimulated expression of the *lux* system by iron-limiting growth conditions requires cAMP and CRP.

B. Gene transfer system for *V. fischeri*

Substantial progress has also been made on development of a gene transfer system for *V. fischeri*. We have tentatively identified a replicon that has the potential of serving as a suicide vector in *V. fischeri* and another replicon that stably replicates in *V. fischeri* under continued selection for antibiotic resistance. The development of a gene transfer system will facilitate our plans for transposon mutagenesis and gene replacement and complementation studies in *V. fischeri*.

WORK PLAN (Year 2):

1. Role of autoinducer in *luxR* negative autoregulation. Generation and screening of putative *luxI* mutants will be continued. Upon confirmation that we have *luxI* mutations by phenotypic screening and DNA sequencing, plasmids containing those mutations will be used in the complementation system described above to determine if the repression of transcription from the *luxR* promoter by the LuxR protein requires, or is stimulated by, autoinducer. This work may also help resolve questions regarding a possible dual role for the *luxI* gene product, that of autoinducer synthesis and that of *luxR* regulation.

2. Mechanism for cAMP-CRP control over iron regulation of luminescence. This work will be carried out in *E. coli* because of the availability of appropriate mutants. Conditions for iron restriction of growth of *E. coli* that result in a stimulation of luminescence and luciferase synthesis analogous to that seen in *V. fischeri* will be defined. The requirement for cAMP and CRP in iron regulation of luminescence will be confirmed using Δ*cya* and Δ*crp* mutants of *E. coli* transformed with the *lux* plasmid pPD201 (intact *lux* genes). During iron-limited growth of the mutants, the effect of cAMP and CRP on transcription from the *luxR* and *lux* operon promoters will be determined using the Mu dI1734 *lacZ*::*luxR* fusion plasmid, pPD313. Various additional regulatory mutants of *E. coli* will be examined to attempt to determine the mechanism for cAMP-CRP control over iron regulation of luminescence.

3. Gene transfer system for *V. fischeri*. A major objective for Year two is to continue the development of methods for moving DNA into *V. fischeri*. This work will include optimizing the efficiency of getting the DNA into *V. fischeri* cells, confirming insertion of foreign DNA into the *V. fischeri* genome, and constructing appropriate vectors for complementation and gene replacement studies. As it develops the procedures will be used initially to introduce random mutations into the chromosome, and later will be used to construct specific *lux* gene and other mutations. The two primary goals of this work are to enable us to examine the details of *lux* gene regulation in *V. fischeri*, using the results and model developed with *E. coli* as a guide, and to initiate studies leading to the identification and analysis of other functions involved in the symbiosis.

INVENTIONS:

none

PUBLICATIONS AND RESEARCH PRESENTATIONS:

1. A paper reporting results of studies on cAMP-CRP control of *lux* gene expression in *E. coli* is in press.

   Dunlap, P. V. and E. P. Greenberg (1988) Control of *Vibrio fischeri lux* gene transcription by a cylic AMP receptor protein-LuxR protein regulatory circuit. J. Bacteriol. 170:000-000 (in press).

2. A manuscript describing the isolation, characterization, and control of luminescence in *V. fischeri* Cya and Crp mutants has been prepared and submitted.

   Dunlap, P. V. Regulation of luminescence by cyclic AMP and iron in Cya and Crp mutants of *Vibrio fischeri*. (Submitted 8/88 to J. Bacteriol.)

3. A preliminary report on the regulation of the *V. fischeri luxR* gene was presented at the New Mexico Branch, Amer. Soc. Microbiol. Ann. Meet., Albuquerque, NM (October 30-31).

   Dunlap, P.V. (1987) Regulation of *Vibrio fischeri lux* gene expression in *Escherichia coli*: role of cAMP-CRP in *luxR* transcriptional activation and negative autoregulation.

4. A preliminary report of studies with the *V. fischeri* Cya and Crp mutants was presented at the Annual Meeting of the American Society for Microbiology in Miami Beach, Florida on May 12.

   Dunlap, P.V. (1988) Control of luminescence in *cya*-like and *crp*-like mutants of *Vibrio fischeri*. Abstract #H177, 88th Ann. Meet. Amer. Soc. Microbiol. (Miami Beach, FL, 8-13 May), pg. 174.

5. An invited seminar was presented to the Department of Biology, Woods Hole Oceanographic Institution, Woods Hole, MA on June 16, 1988.

"Cyclic AMP control of luminescence in the symbiotic bacterium *Vibrio fischeri*."

TRAINING ACTIVITIES AND PERSONNEL:

Ms. Joanne Ray, a research assistant (female caucasian, U.S. citizen) and Mr. Ulrich Mueller, an undergraduate student (male caucasian, citizen of the Federal Republic of Germany, permanent U.S. resident) are working on this project.

AWARDS AND FELLOWSHIPS:

none